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July 11, 2002

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Commissioner for Patents
Washington, D.C. 20231

Re: U.S. Utility Patent Application
Appl. No. 10/058,825; Filed: January 30, 2002
For: **Modified Plants**
Inventor: Roderick John SCOTT
Our Ref: 0623.1160001/LBB/GLL
Art Unit: 1632

Sir:

Transmitted herewith for appropriate action are the following documents:

1. Submission of Certified Copy of 35 U.S.C. § 119(a)-(d) Priority Document In Utility Application;

2. A certified copy of Great Britain Appl. No. 9918061.4; and

3. One return postcard.

It is respectfully requested that the attached postcard be stamped with the date of filing of these documents, and that it be returned to our courier. In the event that extensions of time are necessary to prevent abandonment of this patent application, then such extensions of time are hereby petitioned.

The U.S. Patent and Trademark Office is hereby authorized to charge any fee deficiency, or credit any overpayment, to our Deposit Account No. 19-0036.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

Gaby L. Longsworth

Gaby L. Longsworth
Agent for Applicant
Registration No. 47,756

LBB/GLL/eaf
Enclosures

SKGF_DC1:16079.1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:
Roderick John SCOTT
Appl. No. 10/058,825
Filed: January 30, 2002
For: **Modified Plants**

Confirmation No. 2437
Art Unit: 1632
Examiner: To be assigned
Atty. Docket: 0623.1160001/LBB/GLL

Submission of Certified Copy of 35 U.S.C. § 119(a)-(d) Priority Document In Utility Application

Commissioner for Patents
Washington, D.C. 20231

Sir:

Submitted herewith is a certified copy of Applicant's U.S.C. § 119(a)-(d) priority document, to perfect the claim to priority filed on January 30, 2002.

Country	Priority Document Appl. No.	Filing Date
Great Britain	9918061.4	July 30, 1999

Prompt acknowledgment of this submission is respectfully requested.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

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Dated 1 May 2002

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2. Patent application number
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9918061.4

30 JUL 1999

3. Full name, address and postcode of the or of each patent applicant *(underline all surnames)*

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UK

THE PATENT OFFICE
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30 JUL 1999
LONDON

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation

79815700\

4. Title of the invention

MODIFIED PLANTS

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

KILBURN & STRODE
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Patents ADP number *(if you know it)*

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a) any applicant named in part 3 is not an inventor, or

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Signature: Kilburn & Strode

Date: 30 July 1999

12. Name and daytime telephone number of person to contact in the United Kingdom

MR PAUL CHAPMAN
Tel: 0171-539 4200

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MODIFIED PLANTS

The present invention relates to methods for controlling endosperm size and development in plants. The invention also relates to nucleic acid constructs for use in such methods, as well as modified plants *per se*.

Yield in crop plants where seed is the harvested product is usually defined as weight of seed harvested per unit area (Duvick, 1992). Consequently, individual seed weight is regarded as a major determinant of yield. Most monocotyledonous plants e.g. maize, wheat, (see Esau, 1965) produce albuminous seeds - that is, at maturity they contain a small embryo and a relatively massive endosperm. Consequently, in monocotyledonous plants, the endosperm represents a significant component of seed yield. Endosperms accumulate and store diverse substances, including starch, proteins, oils and fats.

Therefore, in monocotyledons increasing the size of the endosperm or its ability to accumulate storage products is likely to increase individual seed weight and perhaps total yield.

Endosperms are utilised commercially in diverse ways, either indirectly as part of the whole seed or directly following their purification, or the purification of certain of their constituents. Hence endosperms may represent either a proportion or the entire commercial value of a crop.

Examples of indirect usage include fodder maize and whole wheat flour. An example of direct usage of the complete endosperm is in the production of white flour for bread-making. Finally, maize oil represents an example of the utilisation of a constituent of the endosperm, but there are many others.

In contrast to monocotyledons, most dicotyledonous plants, e.g. oil seed rape, soybean, peanut, *Phaseolus vulgaris* (e.g. kidney beans, white beans, black beans),*Vicia faba* (broad bean), *Pisum sativum* (green pea), *Cicer aeietinum* (chick pea), and *Lens culinaris* (lentil) produce exalbuminous seeds - that is, mature seeds lack an endosperm. In such seeds the embryo is large and generally fills most of the volume of the seed, and accounts for almost the entire weight of the seed. In exalbuminous seeds the endosperm is ephemeral in nature and reaches maturity when the embryo is

small and highly immature (usually heart/torpedo stage). Commonly embryo development depends on the presence of the endosperm, which is generally accepted to act as a source of nutrition for the embryo.

Scott *et al* (1998) showed that the size of the endosperm in terms of the number of endosperm cells at maturity in the dicotyledonous plant *Arabidopsis thaliana*, a close relative of oil seed rape (*Brassica napus*), is positively correlated with the weight of the mature seed. Plants that developed seeds with **80%** smaller endosperms (average = 80 nuclei) compared to controls (mean of 2x-2x (diploid plant crosses) and 4x-4x (tetraploid plant crosses) = 400 nuclei) produced seeds that were **46%** smaller (in weight terms = 14μg) than the controls (mean of 2x-2x and 4x-4x = 30μg). In contrast, plants that developed seeds with **160%** bigger endosperms (average = 640 nuclei) compared to controls (mean of 2x-2x and 4x-4x = 400 nuclei) produced seeds that were **180%** larger (in weight terms = 54μg) than the controls (mean of 2x-2x and 4x-4x = 30μg). *Arabidopsis* seed in common with most other dicotyledonous seed is composed almost entirely of embryo. Hence the change in seed weight is almost completely due to a change in embryo weight.

Consequently, modifying endosperm size, in terms of the number of cells at maturity, has a dramatic impact on seed weight in seeds that do not contain endosperm at maturity. Without being bound by the following, one reasonable hypothesis is that a larger endosperm accumulates a greater quantity of reserves from the seed parent, perhaps by acting as a stronger "sink". These reserves then provide more resources for utilisation by the growing embryo, resulting in a larger seed. Alternative mechanisms might operate, however.

The seeds of dicotyledons, like those of monocotyledons are utilised in diverse ways. For example, pulses such as soybean, peanut, *Phaseolus vulgaris* (e.g. kidney beans, white beans, black beans),*Vicia faba* (broad bean), *Pisum sativum* (green pea), *Cicer aeietinum* (chick pea), *Lens culinaris* (lentil) are important world crops that are used directly for animal and human consumption. Seeds of oil rape, sunflower and linseed are processed to produce oils.

Clearly, despite the differences in the structure of monocot and dicot seeds, particularly with respect to the presence or absence of endosperm in mature seeds, the size of the endosperm is an important factor in determining individual seed weight, and therefore potentially total crop yield in plants where seed is the economic harvest. Indeed, Hannah and Greene (1998) showed that maize seed weight is dependent on the amount of endosperm ADP-glucose pyrophosphorylase, the enzyme responsible for supplying substrate for starch synthesis.

However, there is some evidence that an increase in seed weight is associated with a reduction in seed number in many breeding populations. Consequently, increasing individual seed size may not result in an increase in total yield. While maize breeding programmes have been successful and genetic improvement has played a significant role in increased maize yields, the genetic component to yield has led to only a doubling of this parameter since the 1930s (Duvick, 1992). The increase in maize yields is currently less than 1% per year.

The genetic basis for the resistance to increased seed weight encountered in conventional breeding programmes is not understood. However, Giroux et al (1996) showed that a single gene mutation in the endosperm specific gene *shrunken2* of maize resulted in a seed weight increase of 11-18% without a reduction in seed number. This suggests that yield improvements are possible in a plant with a long history of intensive and successful breeding efforts, and may therefore be generally achievable. Similarly, Roekel et al. (1998) showed that introduction of the *tzs* gene into *Brassica napus* results in a significant increase in seed yield accounted for by increased seed number per silique and increased seed weight.

There is also evidence that seed size (weight) is positively correlated with a number of components of "seed quality " such as percent germination (Schaal, 1980: Alexander and Wulff, 1985; Guberac et al, 1998); time to emergence (Winn, 1985; Wulff, 1986); durability (survival under adverse growing conditions) (Krannitz et al, 1991; Manga and Yadav,1995); growth rate (Marshall, 1986) and yield (Guberac et al, 1998). Seed quality is an important factor in the cost of production of commercial seed lots since these must be tested before sale. Consequently, increasing total seed weight, even

without increases in total seed yield may have economic benefits through improvements in seed quality.

Conversely, there may be instances where reduced seed size is advantageous, for example in crops such as strawberry and grape where the product is the fruit rather than the seed.

We have recently demonstrated (Scott et al., 1998) that hybridising *Arabidopsis* plants of different ploidies has reproducible and dramatic effects on the weight of progeny seed. For example, an interploidy cross between a diploid (2x) seed parent and a tetraploid (4x) pollen parent (2x-4x) results in seed which is 240% larger than 2x-2x seed. Conversely, 4x-2x crosses result in a reduced seed size (60% of 2x-2x). Analysis of endosperm development in these F1 seed reveals a clear correlation between final seed size and the size of the endosperm. In common with most dicots, endosperm is not present in the mature *Arabidopsis* seed but is required to nourish the developing embryo. Therefore, increased endosperm size translates into increased seed size by increasing embryo size, presumably by accumulating and then supplying increased nutrition.

In wild type 2x-2x crosses the endosperm is triploid and is formed by the fertilisation of a pair of fused haploid polar nuclei of maternal origin with a haploid sperm of paternal origin. Consequently, there is a 2:1 ratio of maternal to paternal genomes in the normal endosperms. An excess of paternal genomes in the endosperm, e.g. as a result of a 2x-4x cross, causes increased endosperm proliferation (hyperplasia). An excess of maternal genomes in the endosperm (4x-2x crosses) has the opposite effect: decreased endosperm proliferation (hypoplasia).

Scott et al (1998) explain these observations in terms of the genomic imprinting (inactivation) of genes that contribute to endosperm vigour, either positively or negatively. Accordingly, paternal gametes have an overall positive effect on endosperm growth because genes that inhibit endosperm growth or functionality are imprinted, whilst genes that have a positive effect escape imprinting and are active in the endosperm. Adding more paternal genomes into the endosperm via a tetraploid pollen parent therefore increases the number of stimulatory genes resulting in a larger endosperm. Maternal genomes have the opposite effect. Importantly, imprinting effects have been recorded in a

wide range of plant species including maize and brassicas. In mammals, a number of genes that influence foetal growth (typically expressed in the placenta) also exhibit uniparental expression due to imprinting during gametogenesis. Extra doses of these genes also have dramatic effects on embryo size.

Hybridisation is recognised as an important process for producing offspring having a combination of desirable traits from both parents. Hybridisation may be interspecific or intraspecific. Interspecific hybridisation is used for introducing desirable traits such as disease resistance into crop species. However, the ability to make successful sexual crosses is frequently restricted to closely related species because of the existence of a variety of prefertilisation and postfertilisation reproductive barriers (see Stoskopf, Tomes and Christie, 1993). One type of postfertilisation barrier is associated with poor or disrupted endosperm development (postfertilisation endosperm development barrier), which results in inviable seed (see Ehlenfeldt and Ortiz, 1995). Endosperm failure in unsuccessful crosses is due to the operation of a genetically determined system known as endosperm dosage (Haig and Westoby, 1991). Endosperm dosage is a form of genomic imprinting. The removal of the endosperm dosage barrier to sexual interspecific hybridisation would have economic benefits, since non-sexual techniques for hybridisation e.g. somatic hybridisation are costly and difficult.

The endosperm dosage system may also prevent intraspecific hybridisation where the parents are of different genomic constitutions (ploidies) (Haig and Westoby, 1991).

There is currently considerable research effort to develop transgenic technologies (see Koltunow et al., 1995) to introduce apomixis into crop species. In natural apomictic plant species 2n seed is produced without fertilisation of the egg. The genetic constitution of the embryo is therefore identical to that of the seed parent. The economic benefits of introducing an apomixis system into crop species include true breeding F1 hybrids. Currently, F1 hybrid seed is produced annually by hybridising two genetically distinct parents in a labour intensive and costly process. True breeding (apomictic) F1 hybrids could be propagated for sale without the hybridisation step. The removal of this step would potentially therefore reduce production costs.

An essential aspect of apomixis is that the embryo is derived from a cell with an unreduced (2n) number of chromosomes. In natural apomicts this is achieved by modifying meiosis (meiotic reconstitution) such that 2n gametes are produced, or deriving the embryo from a somatic cell with the 2n number of chromosomes. Irrespective of the origin of the embryo the endosperm is invariably derived via meiosis which is either restitutional or reductional. In the former case the two polar nuclei, which upon fertilisation produce the endosperm, are *2n* and in the later case *n*. Given that natural apomicts utilise endosperms generated in this way it is likely to be the case for genetically engineered apomictic crop plants.

A potential problem in the development of apomictic crop species, given this likely dependency on 'sexual endosperms' (formed by fertilisation), is ensuring the successful development of the endosperm, since the endosperm is required to nourish the embryo or itself represents the principal economic harvest. One barrier to endosperm development is the endosperm dosage system. In species with an endosperm dosage system the ratio of maternal to paternal genomes in the endosperm is 2:1. Deviation from this ratio results in endosperm abortion and seed lethality (Haig and Westoby, 1991). Natural apomicts have adopted a number of strategies to ensure endosperm development. A few species (autonomous apomicts) develop a gynogenetic endosperm (maternal) in the absence of fertilisation of the polar nuclei. The majority however, retain fertilisation of the polar nuclei and maintain a 2:1 genomic ratio by modification of either male meiosis (to produce unreduced gametes) or the fertilisation process e.g. fertilisation involves only 1 polar nucleus. Still other species successfully deviate from the genomic 2:1 ratio.

For engineered apomixis the most attractive solution for ensuring endosperm development is the provision of autonomous endosperm development. Solutions involving fertilisation of the polar nuclei are likely to complicate the delivery of apomixis, for example by necessitating the introduction of a mechanism to prevent fertilisation of the "egg" or the need to devise ways to produce 2n male gametes, or by some other means ensure a 2:1 genomic ratio.

One approach to developing an autonomous apomict involves the induction and isolation of mutant genes that condition endosperm development in sexual species without fertilisation. Extensive

screening efforts in *Arabidopsis* met with limited success having identified several mutant genes that condition only limited endosperm development in the absence of fertilisation (Ohad et al., 1996; Chaudhury et al., 1997; Ohad et al., 1999; Kiyosue et al., 1999; Luo et al., 1999). One potential explanation is that these mutations trigger endosperm development but do not overcome the effects of the endosperm dosage system. Endosperms in the mutants would have a genetic constitution of 2 maternal:0 paternal genomes, which deviates from the normal 2:1 genomic ratio. Significantly, Scott et al, 1998, recently showed that *Arabidopsis* possesses a dosage system capable of causing seed abortion where the ratio of parental genomes in the endosperm deviates significantly from 2:1.

The interploidy cross effect on seed size, the postfertilisation endosperm development barrier to interspecific hybridisation and the barrier to autonomous endosperm development are all explicable in terms of genomic imprinting.

In mammals, a number of genes that influence foetal growth (typically expressed in the placenta) exhibit uniparental expression due to genomic imprinting during gametogenesis. Extra doses of these genes can have dramatic effects on embryo size (Solter, 1998). Genomic imprinting also prevents the development of gynogenetic or androgenetic (two paternal genomes, no maternal genome) embryos (Solter, 1998).

In mammals, genes selected for imprinting are maintained in an inactive state by DNA methylation. The enzyme responsible is DNA methyltransferase (MET) which is encoded by a single gene. Mice embryos containing an inactive DNA methyltransferase gene die at an early developmental stage and express both parental copies of genes that are normally imprinted (i.e. uniparentally expressed) (Li et al, 1993). This demonstrates the involvement of DNA methyltransferase in genomic imprinting and a requirement for imprinting in normal development.

In plants the imprinting mechanism is unknown. However, plant genomes contain relatively large amounts of the modified nucleotide 5-methylcytosine (Gruenbaum et al, 1981). Despite evidence implicating cytosine methylation in plant epigenetic phenomena, such as cosupression and inactivation of transposable elements (Napoli et al, 1990; Bender et al, 1995; Brutnell and Dellaporta,

1994, Martienssen et al ., 1995; Matzke and Matzke, 1995) the role of cytosine methylation in plant developmental processes and genomic imprinting remains unclear.

To date three different genes have been found that may be imprinted in the maize endosperm: tubulin (Lund et al 1995), a storage protein regulator gene *dzr* (Chaudhuri, and Messing, 1994) and the *r* gene transcription factor that regulates anthocyanin biosynthesis (Kermicle and Alleman, 1990). In each case, the maternally inherited allele is undermethylated, over-expressed or both, whereas the paternally inherited allele is more methylated or has a reduced level of expression.

In *Arabidopsis, ddm* mutants (decrease in DNA methylation) have been isolated with reduced levels of cytosine methylation in repetitive sequences, although the mutations do not result in any detectable change in DNA methyltransferase activity (Vongs et al., 1993; Kakutani, 1995). After several generations of self pollination, *ddm* mutants exhibit a slight delay (1.7 days) in flowering, altered leaf shape, and an increase in cauline leaf number (Kakutani, et al, 1995). Repeated self pollination of *ddm* mutant plants does however result in the appearance of severe developmental abnormalities (Kakutani et al, 1996).

Arabidopsis plants expressing a DNA methyltransferase 1 (Met1) antisense (Met1as) gene contain reduced levels of DNA methyltransferase activity and a correspondingly reduced level of general DNA methylation (Finnegan et al, 1995; Ronemus et al., 1996). In contrast to *ddm* mutants, *Arabidopsis* plants expressing a Met1as gene develop various developmental abnormalities at high frequency and without repeated self-fertilisation, including floral abnormalities (Finnegan et al, 1996). WO 98/04725 also reports that *Arabidopsis* plants expressing a Met1as gene alter the rate of development of the plant. The development of the endosperm in *ddm* mutants and plants expressing Met1as has not been reported.

The present invention is based on the unexpected observation that a decrease of about 90% in the amount of methylated DNA present in a plant genome results in the production of gametes, both male and female, that behave in a manner that is consistent with the removal or attenuation of genomic imprinting. This is exemplified by the following experiments:

1. Endosperm development in seeds derived from a cross between a wild type 2x plant, as seed parent, and a 2x Met1as plant as pollen parent (2x-2xMet1as), resembles endosperm development in seeds derived from a 4x-2x interploidy cross (Figures 1 and 3). - the endosperm is small/underdeveloped. The resulting seed is smaller in weight terms than seed from control 2x-2x crosses (Table 1). Hence the male gametes from a Met1as plant behave like a female gamete from a wild type plant. This can be explained by proposing the removal or attenuation of imprinting in the male gamete.

2. Endosperm development in seeds derived from a cross between a 2xMet1as plant, as seed parent, and a wild type 2x plant as pollen parent, strongly resembles endosperm development in seeds derived from a 2x-4x interploidy cross between wild type plants (Figures 1 and 3). - that is , the endosperm is large/overdeveloped. The resulting seed is larger in weight terms than seed from control 2x-2x crosses (Table 1). Hence the female gametes from a 2xMet1as plant behave as a male genome of a normally methylated diploid plant. This can be explained by proposing the removal or attenuation of imprinting in the female gamete.

3. Reciprocal crosses between 2xMet1as and 4x wild type plants result in seed abortion (Figures 1 and 3); consequently seeds derived from these crosses are shrivelled and do not germinate (Table 1). The behaviour of the endosperm in seed generated in these crosses depends on the direction of the cross. Where the 4x plant is the seed parent the endosperm is extremely under-developed and contains very few endosperm nuclei and a very small chalazal endosperm (Figure 1, Table 1). In contrast, where the 4x plant is the pollen parent the endosperm of the resulting seeds is over-developed, and contains many endosperm nuclei and a very well developed chalazal endosperm with many associated chalazal nodules (Figures 1 and 3, Table 1). This outcome resembles those obtained in crosses between 2x and 6x wild type plants which routinely fail to produce viable seed (Figure 3) and display very under- (6x-2x) or over-developed (2x-6x) endosperm depending on the direction of the cross. These crosses represent examples of lethal parental genomic excesses within the endosperm that result form the large disparity between the ploidy level of the respective parents. The similarity between the outcomes and the behaviour of the endosperm in 2xMet1as -4x and 2x-6x

reciprocal crosses can be explained by proposing that male and female gametes derived from 2xMet1as plants behave like gametes of the opposite sex with respect to genomic imprinting. This again strongly suggests that DNA hypomethylation caused by the Met1as gene removes or strongly attenuates genomic imprinting.

4. The behaviour of plants homozygous for the *ddm* mutation in reciprocal crosses with 2x and 4x wild type plants is very similar to that of plants homozygous for the Met1as gene (see Figure 2 and Table 1). This strongly suggests that the basis of the interploidy cross effect is associated with general DNA hypomethylation.

Thus, in a first aspect, the present invention provides a method for the production of modified endosperm which comprises the step of transforming a plant, or plant propagating material, with a nucleic acid molecule comprising one or more regulatory sequences capable of directing expression in the male or female germ line and/or gametes of the resultant plant and one or more sequences whose expression or transcription product(s) is/are capable of altering the the degree of methylation of nucleic acid.

Thus, the present invention provides methods for removing or attenuating genomic imprinting, based on targeting the germ line or gametes with transgenes which alter the methylation pattern of genes within the developing endosperm.

The restriction of imprint removal to one or other sex of gamete is desirable for 3 reasons:

1. To provide for removal of imprinting in a single sex of gamete within an individual plant. This will produce the asymmetry of imprinting that is required to mimic the interploidy cross effect in a self-fertilising plant.

2. To prevent developmental abnormalities that are associated with generalised hypomethylation, such as occurs with the CaMV35S driven Met1 antisense gene.

3. To prevent the attenuation of the interploidy cross effect due to the expression of the hypomethylation gene (Met1as) within the endosperm. Crosses between two 2xMet1as plants result in seed with a slightly increased number of endosperm nuclei and normal seed weight (Table 1), which is most easily explained by proposing that the combination of hypomethylated gametes of both sexes allows normal endosperm development

The important property of the nucleic acid molecule used in the transformation step is that DNA of cells that contribute to one sex of germ line is subject to alteration of the pattern of DNA methylation through the activity of the transgenes. The germ-line is the tissue within the reproductive organs that produces the gametes. In the anthers (stamen) this is the microsporogenous cell tissue and in the pistil (gynoecium) the megasporocyte tissue.

Since the timing of the application of the genomic imprints is currently not known the activity of the regulatory sequences, eg promoters (or fragments of promoters) promoters should be as broad as possible whilst remaining consistent with the principles discussed herein.

As will be described herein, alteration of the methylation of plant gamete DNA can be used to alter endosperm development. The effects can be applied to male or female gametes of the transformed plant. Thus, in a second aspect, the present invention provides a method for the production of modified endosperm which comprises the step of transforming a plant, or plant propagating material, with a nucleic acid molecule comprising one or more regulatory sequences capable of directing expression within the developing gynoecium, especially the cell lineage that gives rise to or comprises the female germ line (megasporocyte tissue), within the ovule of the resultant plant and one or more sequences whose expression or transcription product(s) is/are capable of altering the degree of methylation of nucleic acid.

In this aspect of the invention, the resultant endosperm is larger, and hence the seed produced is heavier. Suitable promoters include the promoter, or regulatory sequences/fragments thereof from the *Arabidopsis* AGL5 gene (Sessions et al., 1998), the *Petunia* FBP7 and FBP11 genes (Angenent et al., 1995; Colombo et al.,1995), *Arabidopsis BEL1* gene (Reiser et al., 1995), *Arabidopsis MEDEA*

(*FIS1*) gene (Grossniklaus et al., 1998; Kiyosue et al., 1999), *Arabidopsis FIS 2* (Kiyosue et al., 1999), *FIE (FIS 3)* (Ohad et al., 1999; Kiyosue et al., 1999), orthologs/homologues of these genes from other species. Other promoters that drive expression that is restricted to cells within the female reproductive organs that contribute to the female germ line would also be suitable. Especially suitable are promoters from gynoecium-specific genes that are first expressed during early gynoecium development, preferably before the differentiation of individual ovules, and which maintain their expression until ovule differentiation is complete (contain egg cell and binucleate central cell)

In a third aspect, the present invention provides a method for the production of modified endosperm which comprises the step of transforming a plant, or plant propagating material, with a nucleic acid molecule comprising one or more regulatory sequences capable of directing expression within the developing stamen, especially the cell lineage that gives rise to or comprises the male germ line (microsporocyte tissue) of the resultant plant and one or more sequences whose expression or transcription product(s) is/are capable of altering the degree of methylation of nucleic acid.

In this aspect of the invention, the resultant endosperm is smaller, and hence the seed is lighter. Suitable promoters include promoter fragments derived from the *Arabidopsis* genes *APETALA3* (Jack et al., 1992; Irish and Yamamoto, 1995), the *Arabidopsis PISTILATTA* gene (Goto and Meyerowitz, 1994), the*Arabidopsis E2* (Foster et al., 1992), the *Arabidopsis APG* (Roberts et al., 1993), homologues/orthologs of these genes from other species. Other promoters that drive expression that is restricted to cells within the male reproductive organs that contribute to the male germ line would also be suitable. Especially suitable are promoters from stamen-specific genes that are first expressed during early stamen development, preferably before the differentiation of individual microsporocytes, and which maintain their expression until stamen differentiation is complete.

Herein, promoters that drive gene expression in cells of the germ line or in cells that represent the direct progenitors of the germ line within either the stamen or pistil and which, when in conjunction with the Met1as gene, produce hypomethylated gametes are referred to as 'germ line' promoters.

Thus, as will be appreciated by the skilled person, the present invention allows for the modification of endosperm such that it is either increased or decreased in size. In addition, the development of the endosperm can be altered such that the modified plants can be used in carrying out intraspecific hybridisation or in engineering apomixis.

In one specific embodiment, the degree of methylation is increased. This can readily be achieved by incorporating one or more sequences encoding one or more methylating enzymes into the transgene. Examples of suitable methylating enzymes include:

i) Methylase 1 (acc nr. C10692);
ii) Methylase 1-like gene (acc. nr. Z97335);
iii) Methylase 2 (acc. nr. AL021711); and
iv) Chromomethylase (acc. nr. U53501);

all from *Arabidopsis*.

In another specific embodiment, the degree of methylation is decreased. This can be achieved in anumber of ways. Firstly, by incorporation of one or more sequences encoding one or more de-methylating enzymes, such as de-methylase (= MeCP2-homologue; see below)(acc. nr. AL021635) into the transgene. Alternatively, the transgene can incorporate sequences which cause down regulation of methylating enzymes already present in the plant. For instance, one can use antisense sequences, eg the Met1as "gene". In addition, it has been found that incorporation of whole or partial copies of an already present gene can result in suppression of gene expression. Thus, the transgene can incorporate additional copies, or partial copies, of genes encoding methylating enzymes already ppresent in the plant. In another alternative, the transgene can incorporate a sequence encoding a ribozyme.

In addition to the methylase genes, there are a number of proteins known or suspected to be involved in the process of genomic imprinting. Altering the rate of expression of those genes in the germ line of either sex can also be used to alter the development of the endosperm in a parent-specific manner.

In the African claw toad Xenopus laevis, the product of the methyl-cytosine binding protein 2 (MeCP2) has been shown to specifically bind to methylated cytosines (Kass et al., 1997; Jones et al., 1998). This protein, of which conserved homologs in mammals also exist, forms a complex at the C-met locus with several other proteins. Amongst these are the transcription-repression mSin3 proteins (Nan et al., 1998; Laherty et al., 1997) and a number of histone deacetylases (HDAC). The activity of the latter genes is presumed to be an important step in the process of anchoring histones to the DNA and hence the formation of heterochromatin and the silencing of genes (reviewed in Razin, 1998 and Pazin and Kadonaga, 1997). The MeCP2-protein may thus constitute the first step in the gene silencing process by guiding the heterochromatin-forming machinery to C-met loci. Interestingly, in contrast with this the protein has also been found to have a de-methylating function in that it removes methyl-groups from cytosine residues (Bhattacharya et al., 1999).

If the homologs of proteins in the C-met binding complex in plants are likewise involved in uniparental gene silencing (imprinting) then inactivation of these genes in the maternal or paternal germ lines would be predicted to mimic the uniparental inactivation of the genes responsible for methylation. In addition, there could be a cumulative effect if more than one gene is inactivated. If for instance inactivation of the MET1 gene by antisense transcription or ds-RNA in one of either germ lines is not complete, then introduction of an additional vector causing inactivation of one of the other components of the imprinting machinery will enhance the effect.

In Arabidopsis, possible homologs of the following genes have been found:

MeCP2 (acc nr. AL021635)
HDAC1/2 (acc. nr. AF014824 & AL035538)
mSIN3 (acc. nr. AC007067_5 & AC002396)
p300: a histone acetylation-gene (acc. nr. AC002986.1 & AC002130.1)

In a fourth aspect, the present invention provides an isolated or recombinant nucleic acid molecule, eg a DNA molecule, which comprises one or more regulatory sequences capable of directing expression in the male or female germ line and/or gametes of a plant and one or more sequences whose expression or transcription product(s) is/are capable of altering the the degree of methylation of nucleic acid. The nucleic acid will normally be employed in the form of a vector and such vectors form a further aspect of the invention.

The vector may be for example a plasmid, cosmid or phage. Vectors will frequently include one or more selectable markers to enable selection of cells transfected or transformed and to enable the selection of cells harbouring vectors incorporating heterologous DNA. Examples of such a marker gene include antibiotic resistance (EP-A-0242246) and glucuronidase (GUS) expression (EP-A-0344029). Expression of the marker gene is preferably controlled by a second promoter which allows expression in cells other than the gametes, thus allowing selection of cells or tissue containing the marker at any stage of regeneration of the plant. The preferred second promoter is derived from the gene which encodes the 35S subunit of Cauliflower Mosiac Virus (CaMV) coat protein. However any other suitable second promoter could be used.

Cloning vectors may be introduced into E. Coli or another suitable host which facilitate their manipulation. DNA in accordance with the invention will be introduced into plant cells by any suitable means. Thus, according to yet a further aspect of the invention, there is provided a plant cell including DNA in accordance with the invention.

DNA may be transformed into plant cells using a disarmed Ti-plasmid vector and carried by agrobacterium by procedures known in the art, for example as described in EP-A0117618 and EP-A-0270822. Alternatively the foreign DNA could be introduced directly into plant cells using a particle gun. This method may be preferred for example when the recipient plant is a monocot.

A whole plant can be regenerated from a single transformed plant cell, thus in a further aspect the present invention provides transgenic plants (or parts of them such as propagating material) including DNA in accordance with the invention. The regeneration can proceed by known methods.

Preferred features for each aspect of the invention are as for each other aspect *mutatis mutandis*.

The present invention will now be described with reference to the following examples, which should not be construed as in any way limiting the invention. The examples are accompanied by the following figures:-

FIGURE 1 - Embryo and endosperm development following crosses with Met1-antisense expressing plants as a parent. Confocal micrographs of Feulgen-stained seeds 4-6 days after pollination. Column 1, embryo; column 2, chalazal endosperm; column 3, peripheral endosperm. Note a paternal excess phenotype (well developed chalazal endosperm, highly proliferated peripheral endosperm) in crosses with a demethylated plant as the mother (row 1, 2) and a maternal excess phenotype (small or absent chalazal endosperm and a poorly developed peripheral endosperm) in crosses with a demethylased plant as the father (row 4, 5). See text for full details.

FIGURE 2 - Embryo and endosperm development following crosses with ddm1-mutant plants as a parent. Confocal micrographs of Feulgen-stained seeds 4-6 days after pollination. Column 1, embryo; column 2, chalazal endosperm; column 3, peripheral endosperm. See text for full details.

FIGURE 3 - Embryo and endosperm development following interploidy and balanced crosses. Confocal micrographs of Feulgen-stained seeds 4-6 days after pollination. Column 1, embryo + peripheral endosperm; column 2, chalazal endosperm. For the 2x-4x and 2x-6x crosses (row 6, 7) the peripheral endosperm is shown in an inset. See text for full details.

FIGURE 4 - Schematic diagram showing the method of construction of pAGL5-bin.

FIGURE 5 - Schematic diagram showing the method of construction of pAP3-bin.

FIGURE 6 - Schematic diagram showing the method of construction of pAGL5-asMET1

FIGURE 7 - Schematic diagram showing the method of construction of pAP3-asMET1

Example 1 The use of gametes from hypomethylated plants (Met1as and ddm) mimics the interploidy cross effect (alters number of endosperm nuclei formed and consequently the weight of mature seed).

Reciprocal interploidy (different ploidy) crosses between diploid (2x), and tetraploid (4x) or hexaploid (6x)*Arabidopsis* plants result in changes to both the size of the endosperm, in terms of the number of endosperm nuclei and volume of the chalazal endosperm, and to the dry weight of mature seeds (see Table 1). This is the interploidy cross effect.

Crosses involving Met1as plants

Intraploidy (same ploidy) crosses between 2x Met1as plants and 2x wild type plants mimic this effect (see Table 1 and Figures 1 and 3). A cross between a 2x Met1as plant as seed parent and a 2x wild type plant as pollen parent produces seeds with an average of 450 endosperm nuclei (an increase of 130% over 2xmet-2xmet cross), a relative increase in chalazal endosperm volume of 75% compared to 2xmet-2xmet seed, and a mature dry weight of 20μg (an increase of 33% compared to seed from 2xmet-2xmet cross) (see Table 1).

A cross between a 2x wild type plant as seed parent and a 2x Met1as plant as pollen parent produces seeds with an average of 200 endosperm nuclei (a reduction of 43% over 2xmet-2xmet cross), a relative decrease in chalazal endosperm volume of 50% compared to 2xmet-2xmet seed, and a mature dry weight of 10μg (a decrease of 32% over a wild type 2xmet-2xmet cross) (see Table 1).

Table 1. Outcomes of control crosses and crosses involving Met1 antisense and *ddm* mutant plants

Cross	Interploidy cross phenotype[1]	Viability of hybrid seed (%)[2]	Maximum number of peripheral endosperm nuclei[3]	Relative volume of chalazal endosperm[4]	Relative change to cellulari-sation time (days)[5]	Seed weight (µg)[6]
2x-2x	NA	95-100	400	1	0	22
4x-4x	NA	95-100	400	2.5	0	36
6x-6x	NA	95-100	300	3.5	0	44
2x-4x	PE	95-100	640	2	+1	54
4x-2x	ME	95-100	80	0.6	-1	14
2x-6x	PE	0[7]	400	6.8	absent	6
6x-2x	ME	0[7]	50	0.2	-1.5	4
2xmet-2xmet	PE	95-100	350	1	0	15
2x-2xmet	ME	95-100	200	0.5	-0.5	10
2xmet-2x	PE	95-100	450	1.75	+0.5	20
2xddm-2xddm	PE	95-100	350	1.25	0	19
2x-2xddm	ME	95-100	250	0.5	-0.5	12
2xddm-2x	PE	95-100	400	2	+0.5	21
4x-2xmet	ME	0[7]	100	0.3	-1.5	3
2xmet-4x	PE	0[7]	740	4.4	> +3	15
4x-2xddm	ME	0[7]	150	0.3	-1.5	5
2xddm-4x	PE	0[7]	680	3.5	> +3	5

NA, not applicable; PE, paternal excess; ME, maternal excess.

1, either paternal (PE) or maternal (ME) excess as defined in Scott et al., 1998. 2, determined by germination on soil. 3, counts done as described in Scott et al., 1998. 4, calculated relative to amount in 2x-2x control cross at heart stage (approx. 5 DAP). 5, expressed relative to 2x-2x control cross (usually 5 DAP). 6, measured as described in Scott et al., 1998. 7, seeds shrivelled.

The presence and (possible) activity of the Met1as gene within the endosperm potentially complicates the interpretation of the data produced in out crosses involving homozygous Met1as plants. In such crosses the endosperm (and embryo) inherit a single copy of the Met1as, either from the seed or pollen parent. If the Met1as is active within the endosperm it may,

1. disrupt endosperm development since Met1as show various vegetative and floral abnormalities associated with the misexpression of certain genes that regulate development (Finnegan, 1996). However, the presence of the Met1as gene does not appear to have this effect since the endosperms of seeds derived from self pollinated Met1as plants appear developmentally normal except for a degree of paternal excess (Figure 1).

2. attenuate the magnitude of the interploidy cross effect, by demethylating and thereby erasing imprints from the genome contributed by the normally methylated parent. The imprints must be maintained and propagated in the endosperm if the interploidy cross effect is to be mimicked. The removal of imprints via the action of the Met1as gene could reactivate imprinted loci such that the endosperm genomes behave as if derived from same ploidy parents.

To demonstrate that the interploidy cross effects described above are due to the effect of the Met1as gene on the imprinting of gametes rather than any effect within the endosperm we present data from crosses involving plants hemizygous (that is carrying a single copy) of the Met1as gene. Such plants show patterns of general DNA demethylation similar to homozygotes. Hence gametes derived from these plants are generated in a hypomethylating environment, but because the plants are hemizygous only 50% of these gametes contain the Met1as gene. This enables gametes to be produced in a demethylating environment which then do not subsequently contribute a Met1as into the endosperm when used in crosses. This allows the effect of removing imprints within the gametes to be evaluated in endosperms that do not contain the Met1as gene.

The results of reciprocal crosses involving hemizygotes and 4x wild type plants are shown in Table 2. Both crosses result in a 1:1 ratio of plump, viable : shrivelled, inviable seed. The shrivelled seeds are assumed to result from lethal parental excess caused by the union of a hypomethylated gamete from the hemizygote and a 2x gamete from the 4x parent. Conversely, the plump seeds are assumed to result from normally methylated gamete from the hemizygote and a 2x gamete from the 4x parent. Met1as plants appear therefore to produce both normally methylated and hypomethylated gametes. The plump seeds produce plants which segregate 1:1 for the Met1as gene. Presumably, the shrivelled seeds also segregate 1:1 for the Met1as gene. This data therefore demonstrates that the presence of the

transgene in the endosperm is not responsible for the lethality phenotype associated with 2xMet1as-4x reciprocal crosses. If this were the case, seeds containing the Met1as gene would not be recovered among the plump, viable seed class.

Crosses involving ddm mutant plants

Table 1 shows that crosses between wild type diploid and wild type tetraploid plants and plants homozygous for the ddm mutation have very similar outcomes to crosses involving plants containing the Met1as gene. The common feature of the ddm mutation and the action of the Met1as gene is that plants containing these genes have highly hypomethylated DNA. This shows that the interploidy cross effect produced in crosses involving gametes derived from ddm and Met1as plants is related to DNA hypomethylation.

The hemizygote data (Table 2) further suggests that the phenomenon involves hypomethylation of the gametes, presumably through the removal of genomic imprints.

Table 2. Outcomes of reciprocal crosses between *Arabidopsis* plants hemizygous for the Met1a gene and wild type 4x plants.

	Mature Seed phenotypes (%)[1]		Seed viability (%)[2]		Proportion viable seeds carrying Met1as gene (%)[3]	Seed weight (μg)[4]	
	Plump seeds	Shrivelled seeds	Plump seeds	Shrivelled seeds		Plump seeds	Shrivelled seeds
4x-2xmetHET	50	50	95-100	0	50	11	2
2xmetHET-4x	50	50	95-100	0	50	23	8

Abbreviations: 2x, wild type diploid plant; 4x, wild type tetraploid plant; 2xmetHET, plant hemizygous for the Met1as gene

1, scored by eye. **2**, determined by germination on soil of seed from mature pods. **3**, determined by PCR analysis on plants germinated from plump seeds. **4**, measured as described in Scott et al., 1998.

Example 2 Construction of expression cassettes that restrict gene expression to either the gynoecium or the stamen.

Example 1 demonstrates that uniparental demethylation can be used to control seed size. However the desired increases in seed size are not obtained in crosses of wild type plants to ddm or 35SMet1as female lines. This is due to the reduced fitness of the 35SMet1as and ddm female lines since demethylation occurs in all plant tissues. In order to reduce and eliminate this effect and to allow seed size changes to be obtained in a single plant it is neccessary to restrict demethylation as much as possible to the germ line.

1. Designing a general female-germ line specific expression vector

An expression vector based on the female-specific AGL5 promoter (Sessions et al (1998)) is constructed as described below. The nos polyA signal sequence is excised from pCaMVNEO (Fromm et al (1986)) as a BamHI, HindIII fragment and cloned between the BamHI and HindIII sites of pBin19 (Bevan 1994) forming pNosterm-bin. A 2.2kb AGL5 promoter is PCRed from Arabidopsis genomic DNA using the primers AGL5F and AGL5R which introduce an EcoRI and a KpnI site at the ends of the AGL5 PCR fragment.

5' CCGAATTCTTCAAGCAAAAGAATCTTTGTGGGAG 3' AGL5F
EcoRI

5' CGGTACCTATAAGCCCTAGCTGAAGTATAAACAC 3' AGL5R
KpnI

The AGL5 PCR fragment is cloned as an EcoRI, KpnI fragment between the EcoRI and KpnI sites of pNosterm-bin forming pAGL5-bin (figure 4).

2. Designing a general male-germ line specific expression vector

An expression vector based on the male-specific *APETALA* (AP3) promoter (Irish and Yamamoto (1995)) is constructed as described below. A 1.7 kb AP3 promoter is PCRed from Arabidopsis genomic DNA using the primers AP3F and AP3R which introduce an EcoRI and a KpnI site at the ends of the AP3 PCR fragment.

5' CCGAATTCAAGCTTCTTAAGAATTATAGTAGCACTTG 3' AP3F
EcoRI

5' GGGTACCTTCTCTCTTTGTTTAATCTTTTTGTTGAAGAG 3' AP3R
KpnI

The AP3 PCR fragment is cloned as an EcoRI, KpnI fragment between the EcoRI and KpnI sites of pNosterm-bin forming pAP3-bin (figure 5).

Example 3: Construction of chimaeric gene fusions between the female (Example 2a) and male (Example 2b) germ-line specific cassettes and the Met1 antisense gene.

Expression of the MET1 gene can be reduced in the female or male germlines by employing techniques known in the art. For example MET1 downregulation can be achieved by expressing antisense MET1 or antisense MET1 fragments or sense MET1or partial sense MET1 or ribozymes directed against MET1 or combinations of the preceding, from promoters expressed in the required germ-line. Below is an example of an antisense MET1 approach.

a) The construction of a female germ-line specific Met1as gene

The MET1 cDNA (accession number is L10692) is 4.7 kb long and is isolated by RT-PCR from Arabidopsis cDNA using the primers MET1F and MET1R.

5' ACTCGAGATTTTGAAAATGGTGGAAAATGGGGC 3' MET1F
XhoI

5' ACCCGGGTGGTTATCTAGGGTTGGTGTTGAGGAG 3' MET1R
SmaI

The resulting MET1 PCR fragment is then cloned as a SmaI, XhoI fragment between the SmaI and SalI sites of pAGL5-bin forming pAGL5-asMET1 (figure 6).

b) The construction of a male germ-line specific Met1as gene

The MET1 PCR fragment fragment is cloned as a SmaI, XhoI fragment between the SmaI and SalI sites of pAP3-bin forming pAP3-asMET1 (figure 7).

Example 4: Introduction of female and male germ-line specific demethylating genes into transgenic plants

Chimaeric genes were introduced via *Agrobacterium*-mediated transformation into wild type diploid *Arabidopsis* using well known techniques.

a) pAGL5Met1as

Transgenic *Arabidopsis* plants containing the pAGL5Met1as gene were vegetatively normal and produced flowers with the normal complement of floral organs.

Arabidopsis containing pAGL5Met1as were pollinated with pollen from wild-type diploid plants or allowed to self pollinate. Endosperm development in the resulting seeds was monitored by confocal microscopy (Scott et al., 1998) and seed weights were measured at maturity. In both cases, endosperms showed a moderate paternal excess phenotype (average maximum endosperm size = 600 nuclei, delayed cellularisation (+1.5 days relative to 2x-2x crosses wild type) and chalazal endosperm hyperplasia) similar to that obtained in 2x-4x crosses between wild type plants (Table 1). This represents an increase in peripheral endosperm size of 150% compared to the average for 2x-2x and 4x-4x crosses (400 nuclei).

The mean weight of mature seed collected from pAGL5Met1as plants was 50µg, compared with a mean of 22µg for 2x-2x seed and 37µg for 4x-4x seed. This represents an increase in seed size of 160% compared to the mean of the 2x-2x and 4x-4x seed weight (30µg).

The germination frequency was comparable to that of seed from 2x-2x wild type crosses - about 95-100%.

The pAGL5Met1as gene could be transformed into other crop species such as B.napus and Zea mays, leading to an increase in seed size and seed quality in the transgenic plants. In this case it is most preferable to use MET1 and AGL5 orthologous sequences from B.napus and Zea mays.

b) pAP3Met1as

Transgenic *Arabidopsis* plants containing the pAP3Met1as gene were vegetatively normal and produced flowers with the normal complement of floral organs.

Arabidopsis containing pAP3Met1as were pollinated with pollen from wild-type diploid plants or allowed to self pollinate. Endosperm development in the resulting seeds was monitored by confocal microscopy (Scott et al., 1998) and seed weights were measured at maturity. In both cases, endosperms showed a moderate maternal excess phenotype (average maximum endosperm size = 80 nuclei, precocious cellularisation (-1.5 days relative to 2x-2x crosses wild type) and chalazal endosperm hypoplasia) similar to that obtained in 4x-2x crosses between wild type plants (Table 1). This represents an decrease in peripheral endosperm size of 80% compared to the average for 2x-2x and 4x-4x crosses (400 nuclei).

The mean weight of mature seed collected from pAP3Met1as plants was 15 g, compared with a mean of 22µg for 2x-2x seed and 37µg for 4x-4x seed. This represents an decrease in seed size of 50% compared to the mean of the 2x-2x and 4x-4x seed weight (30µg).

The germination frequency was comparable to that of seed from 2x-2x wild type crosses - about 95-100%.

The pAP3Met1as s gene could be transformed into other crop species such as *B.napus* and *Zea mays*, leading to an decrease in seed size in the transgenic plants. In this case it is most preferable to use MET1 and AP3 orthologous sequences from B.napus and Zea mays.

Example 5: Promoting interspecific hybridisation

pAGL5Met1as and pAP3Met1as were transformed into *Brassica campestris* and *Brassica oleracea* via standard methods. Reciprocal crosses between the transgenic individuals of the two species yield plump seeds which germinate to give hybrid plants. Crosses between wild type individuals of the two species result in shrivelled seeds which fail to germinate. Hence the two transgenes overcome the normal barrier to interspecific hybridisation. The same genes could be introduced into other species which normally do not hybridise sexually.

Example 6: maternal hypomethylation promotes autonomous endosperm development

In the absence of fertilisation, *Arabidopsis* plants heterozygous for the *fie* mutation (FIE/fie) produce seeds with partial endosperm development (Ohad et al., 1996; 1999; see also Table 3). These 'autonomous' endosperms consist of a severely reduced number of endosperm nuclei (compared to wild type controls) and the endosperm fails to undergo cellularisation. The seed collapses and becomes shrivelled at maturity (Table 3). Consequently, the *fie* mutation conditions only limited endosperm development restricting its utility in the production of autonomous apomictic seed crops or embryoless seed crops. Endosperms produced in plants carrying the Fis1/mea and Fis2 mutations are very similar to those of fie plants, and hence the utility of these genes is also restricted.

Since *fie* endosperms do not contain a paternal genomic contribution one hypothesis is that proper development of the endosperm requires the expression of paternally derived genes that are subject to maternal imprinting.

When plants heterozygous for the *fie* mutation are pollinated with wild type pollen from a 2x wild type plant the ovules carrying the fie allele develop into seeds that abort at heart/torpedo stage, while

ovules carrying the wild type *FIE* allele develop normally (Ohad et al., 1996; 1999; Table 3). The aborted seeds express a strong paternal excess phenotype (Table 3), despite containing only a single paternal contribution. This suggests that a complex situation with respect to imprinting applies within fertilised and unfertilised *fie* endosperms. One hypothesis is that the *fie* mutation lifts imprinting from a proportion of genes normally subject to maternal imprinting: the introduction of a additional paternal genome following fertilisation generates an effective lethal paternal excess such as encountered in a 2x-6x wild type cross (Table 1). The failure of *fie* endosperms to development normally in the absence of fertilisation is also accounted for by this hypothesis, since not all maternally imprinted genes may be derepressed.

Since gametes derived from hypomethylated plants (Met1as and *ddm*) appear to have no or highly attenuated imprinting , and therefore act as gametes of the opposite sex in endosperms, we hypothesised that such gametes in combination with the *fie* mutation would promote complete endosperm development. In the first experiment, we used pollen from a Met1as plant to pollinate a FIE/fie heterozygote and found that 95-100% of the seeds produced were plump and viable. The seeds segregate 1:1 for the FIEFIE:FIEfie genotypes, showing that the fie allele is transmissible through the seed parent in this cross. The FIEFIE seeds display a maternal excess phenotype as expected - endosperm under-development and a reduced seed weight (Table 3), whilst the Fie fie seeds display a moderate paternal excess phenotype. When wild type pollen from a diploid plant is used in this cross, the resulting seeds segregate 1:1 for plump/viable:shrivelled/inviable and the ovules containing the *fie* mutation produce inviable seed since the plump seeds all contain the wild type *FIE* allele (Table 3). Therefore, paternal gametes from Met1as plants appear to rescue fie containing seeds from lethality by reducing the magnitude of the paternal excess phenotype. This supports the hypothesis as outlined above.

In the second experiment we combined the *fie* mutation and the Met1as gene into the same individual (see Table 3). When these plants were emasculated and left unpollinated 50% of the ovules underwent autonomous endosperm development as expected for ovules carrying the fie mutation. Confocal microscopy showed that these seeds contained well developed, cellularised endosperms that resembled endosperm from wild-type plants. The mature seeds were shrivelled, but weighed 20μg. In contrast,

developing ovules of emasculated and unpollinated Fie/fie plants contained very under-developed endosperm that did not cellularise. The mature seeds were shrivelled and weighed 5μg. The production of near wild type endosperm in plants containing both the *fie* mutation and the Metla1as gene shows that the lifting or attenuation of imprinting within the maternal gamete as conditioned by the Met1as gene is sufficient to relieve the developmental block encountered in unpollinated fie ovules. This greatly extends the utility of the autonomous endosperm mutants (fis1, fis 2, fis3, and fie).

Table 3. Enhancement of endosperm development in *fie* mutant seeds by hypomethylation

	Mature Seed phenotypes (%)[1]		Seed viability[2]		Seed weight (g)[3]		Extent of endosperm development (%)[4]	
	Plump seeds	Shrivelled seeds	Plump seeds	Shrivelled seeds	Plump seeds	Shrivelled seeds	Comple	Partial
FIE/fie x 2x	50	50	95-100	0	25	15	50	50[5]
FIE/fie x 2xmet	100	0	95-100	NA	50%= 15 50%= 30	NA	100	0
FIE/fie emasculated	0	100	NA	0	NA	5	0	100[6]
FIE/fie: 2xmetHET emasculated	0	100	NA	0	NA	20	100	0

NA, not applicable; FIE/fie, plant heterozygous for the *fie* mutation; 2x, wild type diploid plant; 2xmet, plant homozygous for the Met1as gene; FIE/fie, 2xmetHET FIE/fie heterozygous line containing a single Met1as antisense gene (introduced by crossing FIE/fie and Met1as and recovering appropriate genotype in the F1).

[1], scored by eye. [2], determined by germination on soil. [3], measured as described in Scott et al., 1998. [4], determined by confocal microscopy as described in Scott et al, 1998; complete corresponds to normal development as occurs in control crosses, partial refers to abnormal development such as a failure to cellularise or develop chalazal endosperm. [5], resembles lethal paternal excess as occurs in 2x-6x crosses [6], as described by Ohad et al, 1999.

Example 7: Production of plants that combine the *fie* mutation and the female germ-line specific demethylating gene, AGL5Met1a

Plants heterozygous for the fie mutation and hemizygous for the pAGL5Met1as gene were generated by making crosses between FIEfie plants as pollen parent and plants homozygous for the pAGL5Met1as gene as seed parent. These plants were vegetatively normal and produced normal flowers. When emasculated 50% of the ovules initiated seed development without fertilisation. Confocal microscopy showed that endosperm development was extensive, resulting in a large (400 nuclei) cellularised endosperm.

The pAGL5Met1as gene could be introduced into crop species, such as *B.napus* and *Zea mays* in which expression of the *FIE* gene, or any of the genes that condition autonomous endosperm development, is suppressed or absent through mutation or the use of transgenic technologies, to produce promote apomixis or embryoless (pseudoapomictic) seed. Preferably the pAGL5Met1as construct contains B.napus or Z.mays MET1 and AGL5 orthologous sequences.

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CLAIMS:

1. A method for the production of modified endosperm, which comprises the step of transforming a plant, or plant propagating material, with a nucleic acid molecule comprising one or more regulatory sequences capable of directing expression in the male or female germ line and/or gametes of the resultant plant, and one or more sequences whose expression or transcription product(s) is/are capable of altering the degree of methylation of nucleic acid.

2. A method for the production of modified endosperm, which comprises the step of transforming a plant, or plant propagating material, with a nucleic acid molecule comprising one or more regulatory sequences capable of directing expression within the developing gynoecium, especially the cell lineage that gives rise to or comprises the female germ line (megasporocyte tissue), within the ovule of the resultant plant, and one or more sequences whose expression or transcription product(s) is/are capable of altering the degree of methylation of nucleic acid.

3. A method for the production of modified endosperm which comprises the step of transforming a plant, or plant propagating material, with a nucleic acid molecule comprising one or more regulatory sequences capable of directing expression within the developing stamen, especially the cell lineage that gives rise to or comprises the male germ line (microsporocyte tissue) of the resultant plant and one or more sequences whose expression or transcription product(s) is/are capable of altering the degree of methylation of nucleic acid.

4. A method as claimed in any one of claims 1 to 3 wherein the one or more regulatory sequences comprise a promoter sequence, or regulatory sequences or fragments therefrom.

5. A method as claimed in any one of claims 1, 2 or 4 wherein the promoter is derived from the *Arabidopsis* AGL5 gene, the *Petunia* FBP7, the *Petunia* FBP11 gene, the *Arabidopsis BEL1* gene, the *Arabidopsis MEDEA* (*FIS1*) gene, the *Arabidopsis FIS 2* gene, the *Arabidopsis FIE (FIS 3)* gene, orthologs/homologues of any of these genes from other species or any promoter that drives expression that is restricted to cells within the female reproductive organs that contribute to the female

germ line, preferably promoters from gynoecium-specific genes that are first expressed during early gynoecium development, preferably before the differentiation of individual ovules, and which maintain their expression until ovule differentiation is complete.

6. A method as claimed in any one of claims 1, 3 or 4 wherein the promoter is derived from the *Arabidopsis* gene *APETALA3,* the *Arabidopsis PISTILATTA* gene, the*Arabidopsis E2* gene, the *Arabidopsis APG* gene, homologues/orthologs of these genes from other species or any promoter that drives expression that is restricted to cells within the male reproductive organs that contribute to the male germ line, preferably promoters from stamen-specific genes that are first expressed during early stamen development, preferably before the differentiation of individual microsporocytes, and which maintain their expression until stamen differentiation is complete.

7. A method as claimed in any one of claims 1 to 6 wherein the size of the endosperm is altered.

8. A method as claimed in any one of claims 1 to 6 wherein development of the endosperm is altered.

9. A method as claimed in any one of claims 1 to 8 wherein the degree of nucleic acid methylation is increased.

10. A method as claimed in claim 9 wherein the nucleic acid molecule includes a sequence encoding a methylating enzyme such as Methylase 1, Methylase 1-like enzyme, Methylase 2 or Chromomethylase of *Arabidopsis*.

11. A method as claimed in any one of claims 1 to 8 wherein the degree of nucleic acid methylation is decreased.

12. A method as claimed in claim 11 wherein the nucleic acid molecule includes a sequence encoding a de-methylating enzyme such as de-methylase (= MeCP2-homologue) of *Arabidopsis*.

13. A method as claimed in claim 11 wherein reduction in nucleic acid methylation is achieved by down-regulation of one or more methylating enzymes present in the plant.

14. A method as claimed in claim 13 wherein the nucleic acid molecule includes a sequence encoding an antisense nucleic acid molecule, a full or partial copy of a methylating enzyme gene already present in the plant or sequence, or a sequence encoding a ribozyme.

15. A method as claimed in claim 14 wherein the nucleic acid molecule includes the sequence of the Met1as "gene".

16. An isolated or recombinant nucleic acid molecule, eg a DNA molecule, which comprises one or more regulatory sequences capable of directing expression in the male or female germ line and/or gametes of a plant and one or more sequences whose expression or transcription product(s) is/are capable of altering the degree of methylation of nucleic acid.

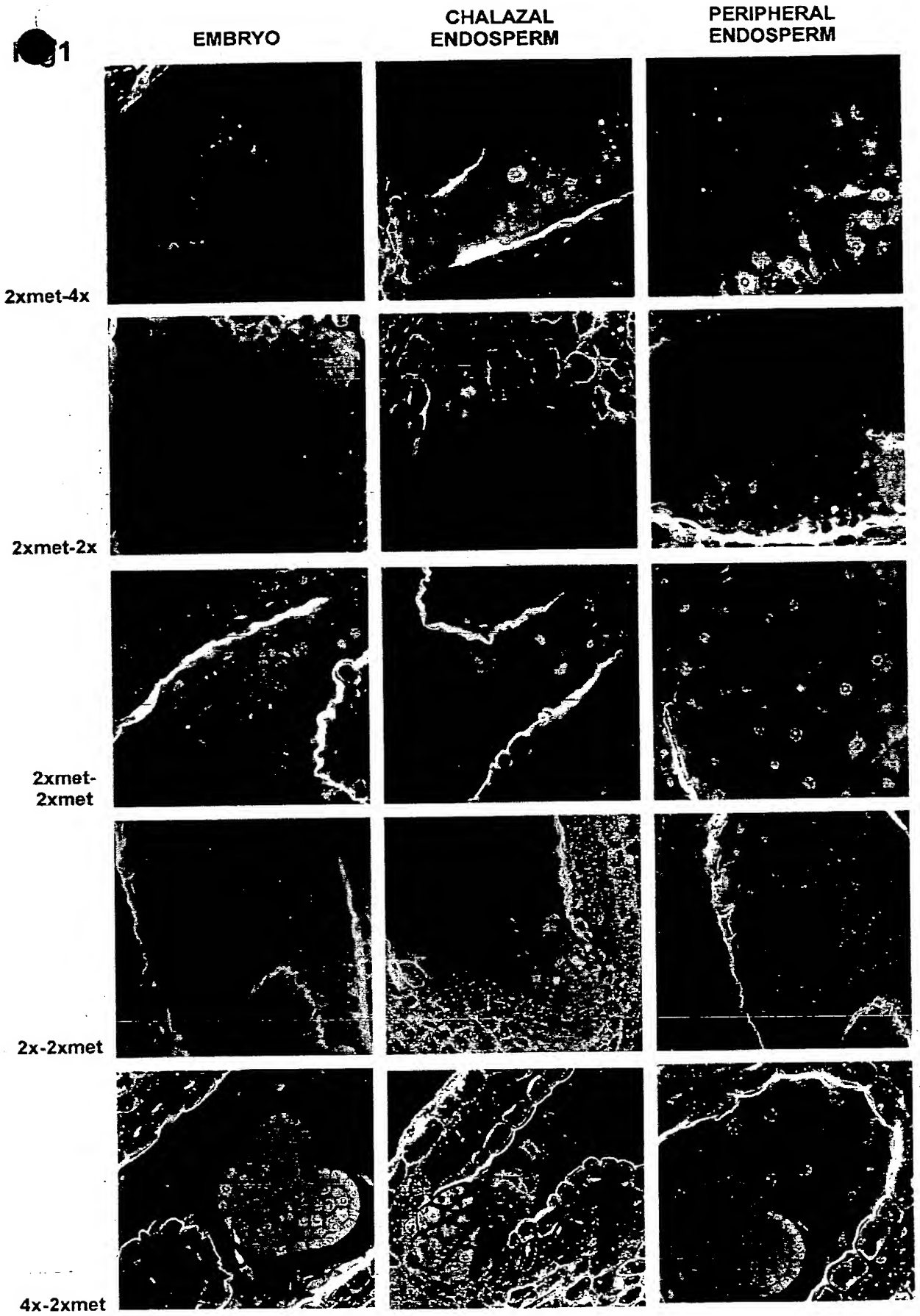
17. A nucleic acid molecule as claimed in claim 16 modified by any one or more of the features defined in any of claims 4 to 15.

18. A nucleic acid molecule as claimed in claim 16 or claim 17 which is in the form of a vector.

19. A plant cell including nucleic acid as defined in any one of claims 16 to 18.

20. A transgenic plant (or parts thereof such as propagating material) including nucleic acid as defined in any one of claims 16 to 18.

Fig 1


EMBRYO
CHALAZAL ENDOSPERM
PERIPHERAL ENDOSPERM
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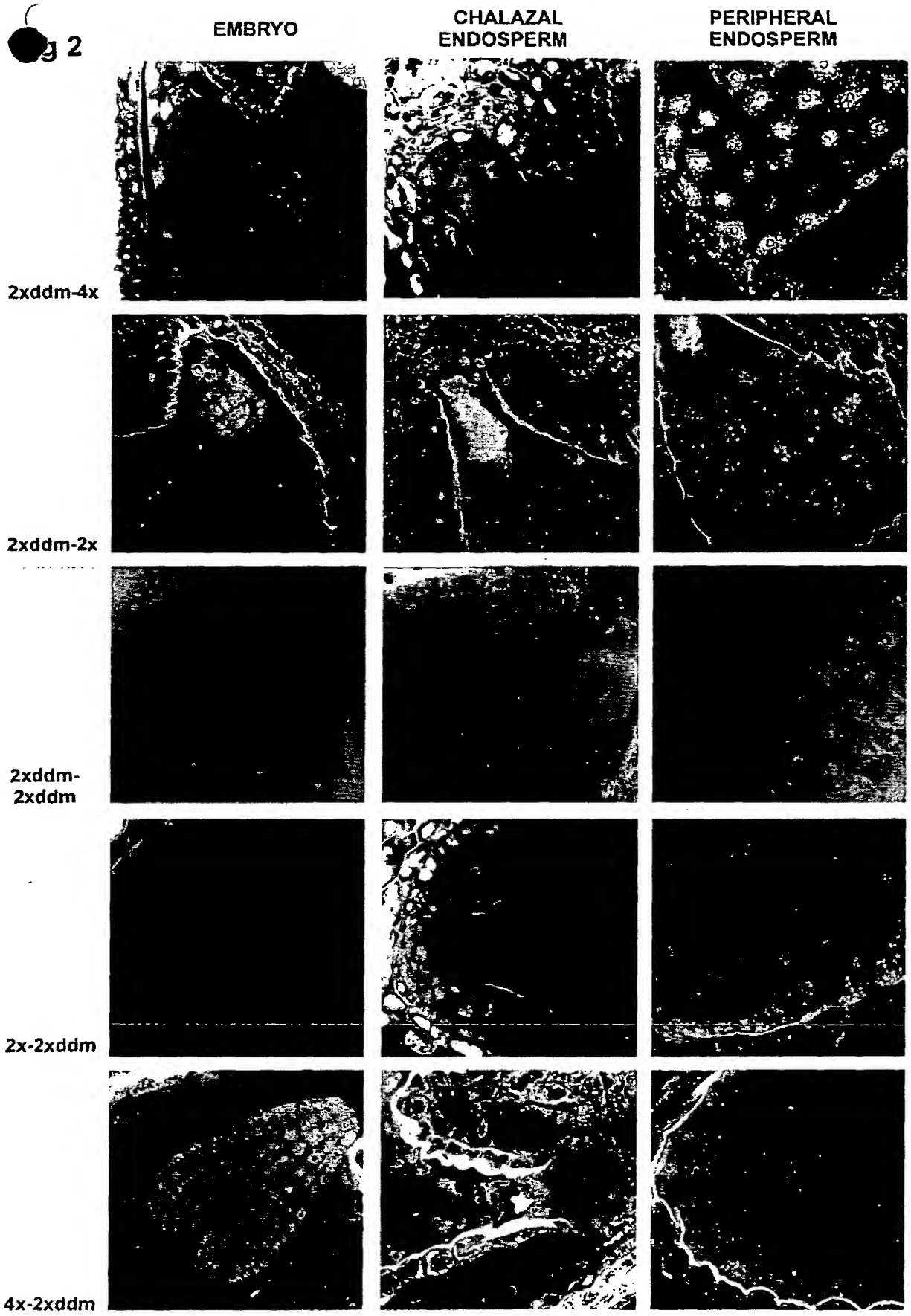
2xmet-2x
2xmet-2xmet
2x-2xmet
4x-2xmet

g 2


EMBRYO
CHALAZAL ENDOSPERM
PERIPHERAL ENDOSPERM
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2xddm-2xddm
2x-2xddm
4x-2xddm

Fig3

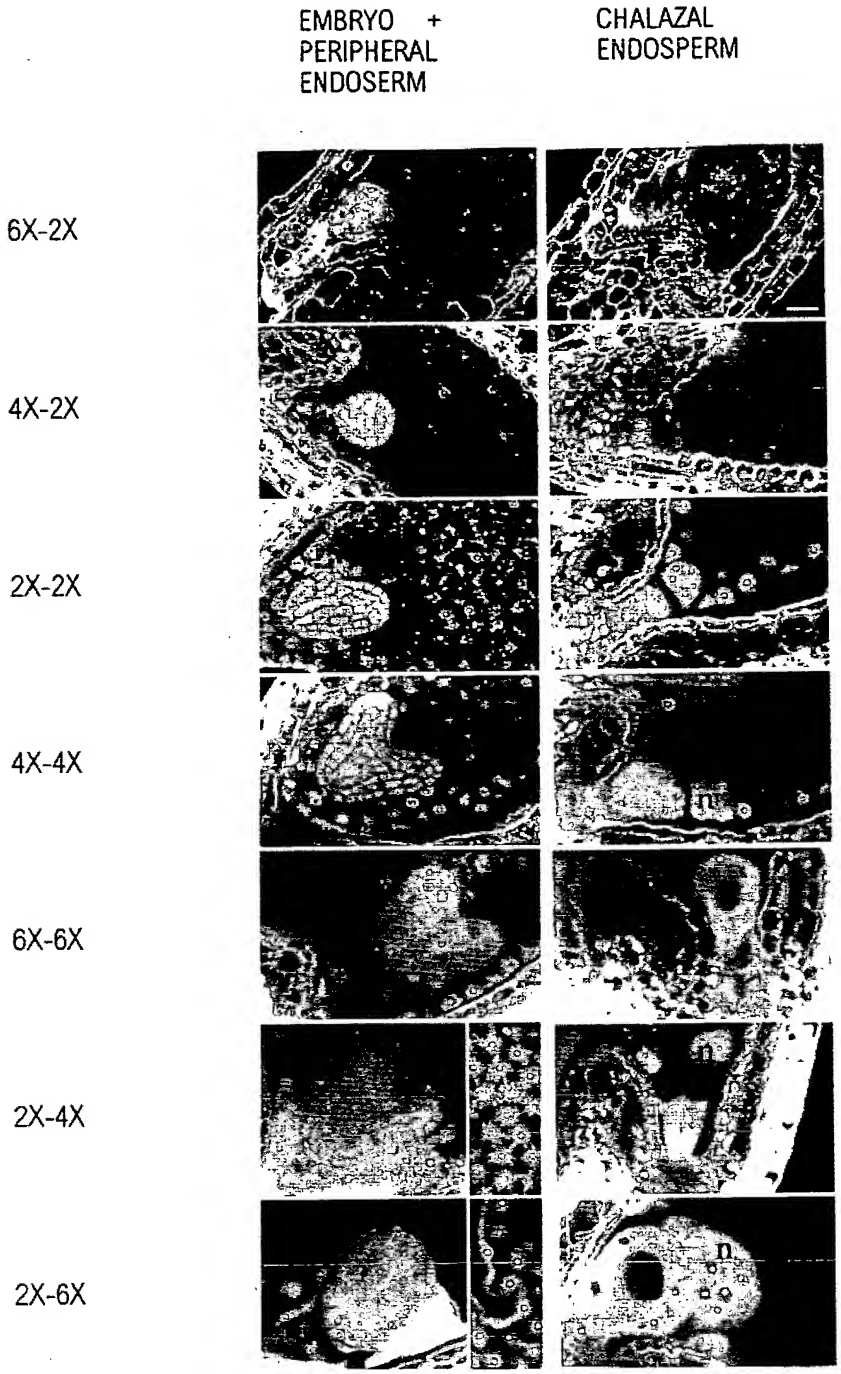

EMBRYO + PERIPHERAL ENDOSERM
CHALAZAL ENDOSPERM
6X-2X
4X-2X
2X-2X
4X-4X
6X-6X
2X-4X
2X-6X
n
n
n

FIGURE 4

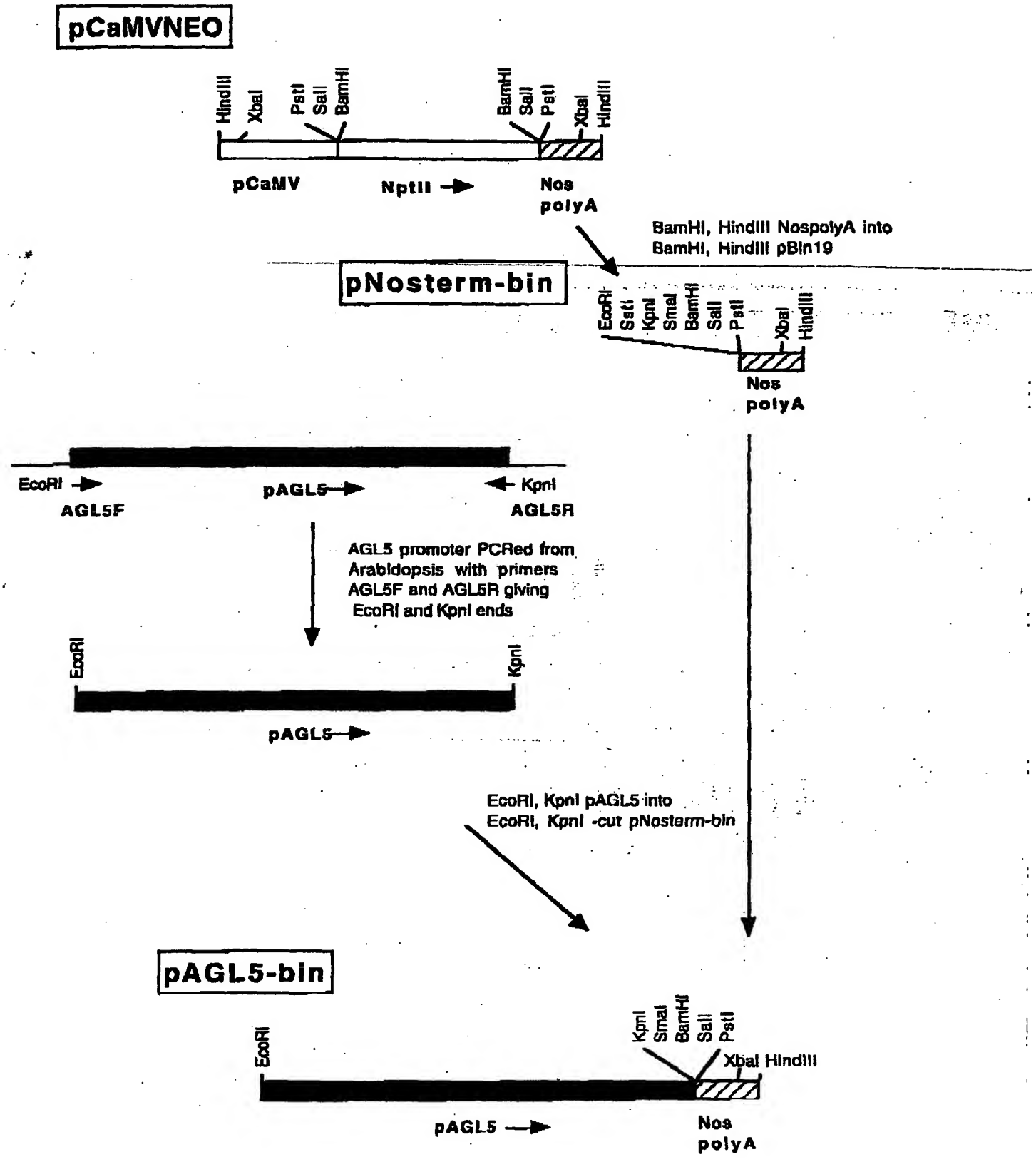
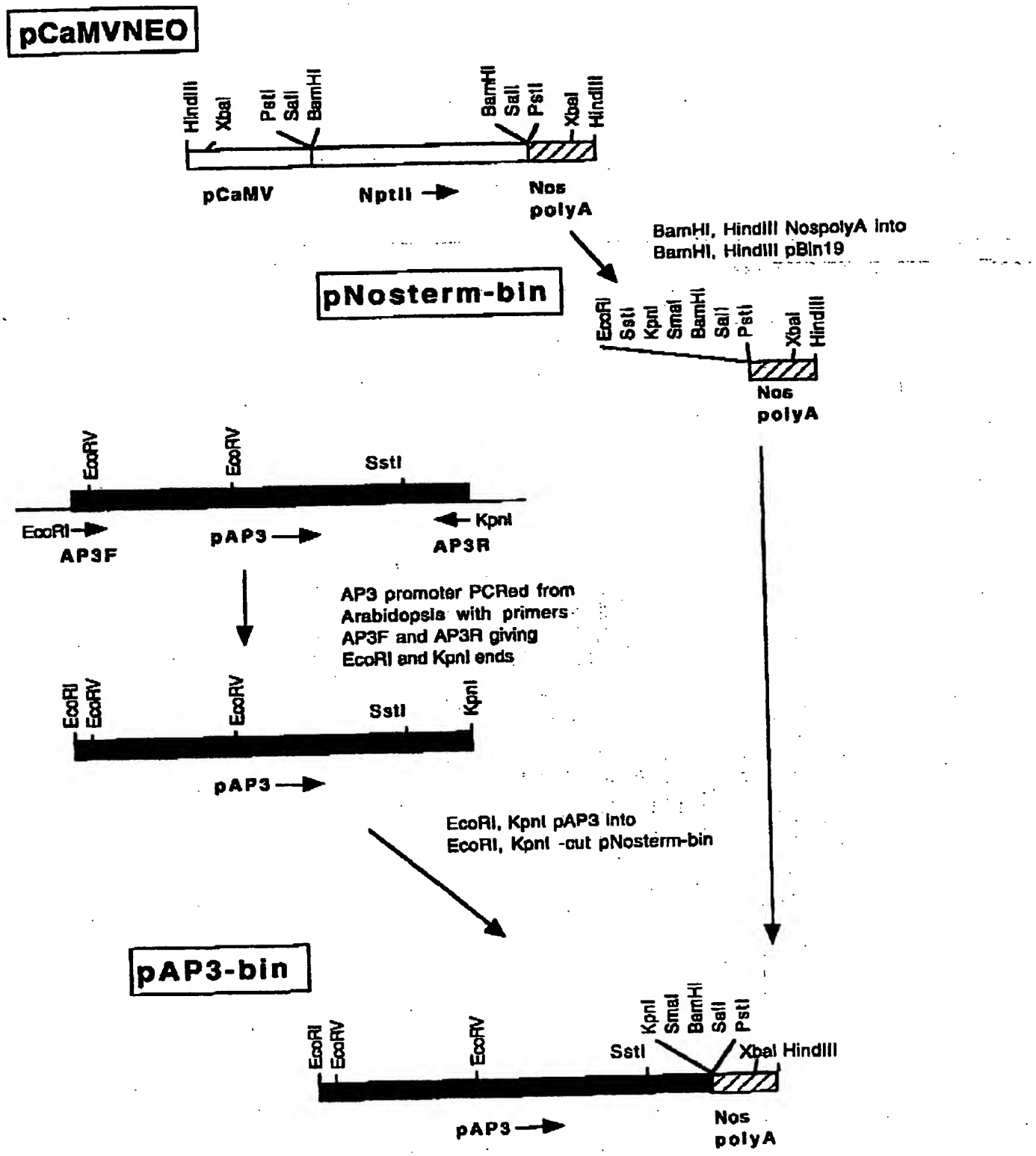

pCaMVNEO
HindIII
XbaI
PstI
SalI
BamHI
BamHI
SalI
PstI
XbaI
HindIII
pCaMV
NptII
Nos polyA
BamHI, HindIII NospolyA into BamHI, HindIII pBIn19
pNosterm-bin
EcoRI
SstI
KpnI
SmaI
BamHI
SalI
PstI
XbaI
HindIII
Nos polyA
EcoRI
AGL5F
pAGL5
KpnI
AGL5R
AGL5 promoter PCRed from Arabidopsis with primers AGL5F and AGL5R giving EcoRI and KpnI ends
EcoRI
KpnI
pAGL5
EcoRI, KpnI pAGL5 into EcoRI, KpnI -cut pNosterm-bin
pAGL5-bin
EcoRI
KpnI
SmaI
BamHI
SalI
PstI
XbaI HindIII
pAGL5
Nos polyA

FIGURE 5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HindIII XbaI PstI SalI BamHI
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pNosterm-bin
EcoRI SstI KpnI SmaI BamHI SalI PstI XbaI HindIII
Nos polyA
EcoRV EcoRV SstI
EcoRI
AP3F
pAP3
KpnI
AP3R
AP3 promoter PCRed from Arabidopsis with primers AP3F and AP3R giving EcoRI and KpnI ends
EcoRI EcoRV EcoRV SstI KpnI
pAP3
EcoRI, KpnI pAP3 into EcoRI, KpnI -cut pNosterm-bin
pAP3-bin
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KpnI SmaI BamHI SalI PstI
SstI
XbaI HindIII
pAP3
Nos polyA

FIGURE 6

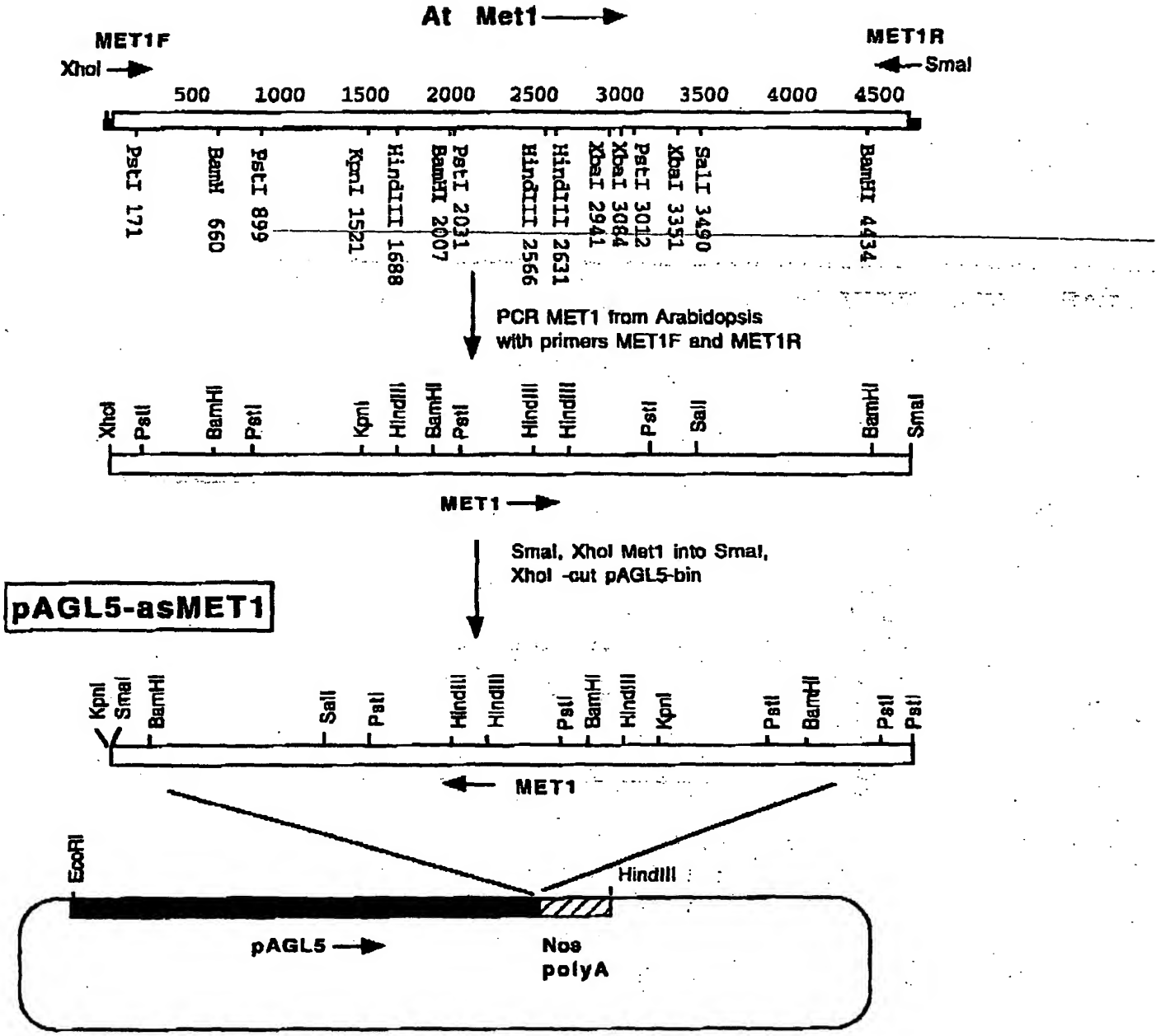
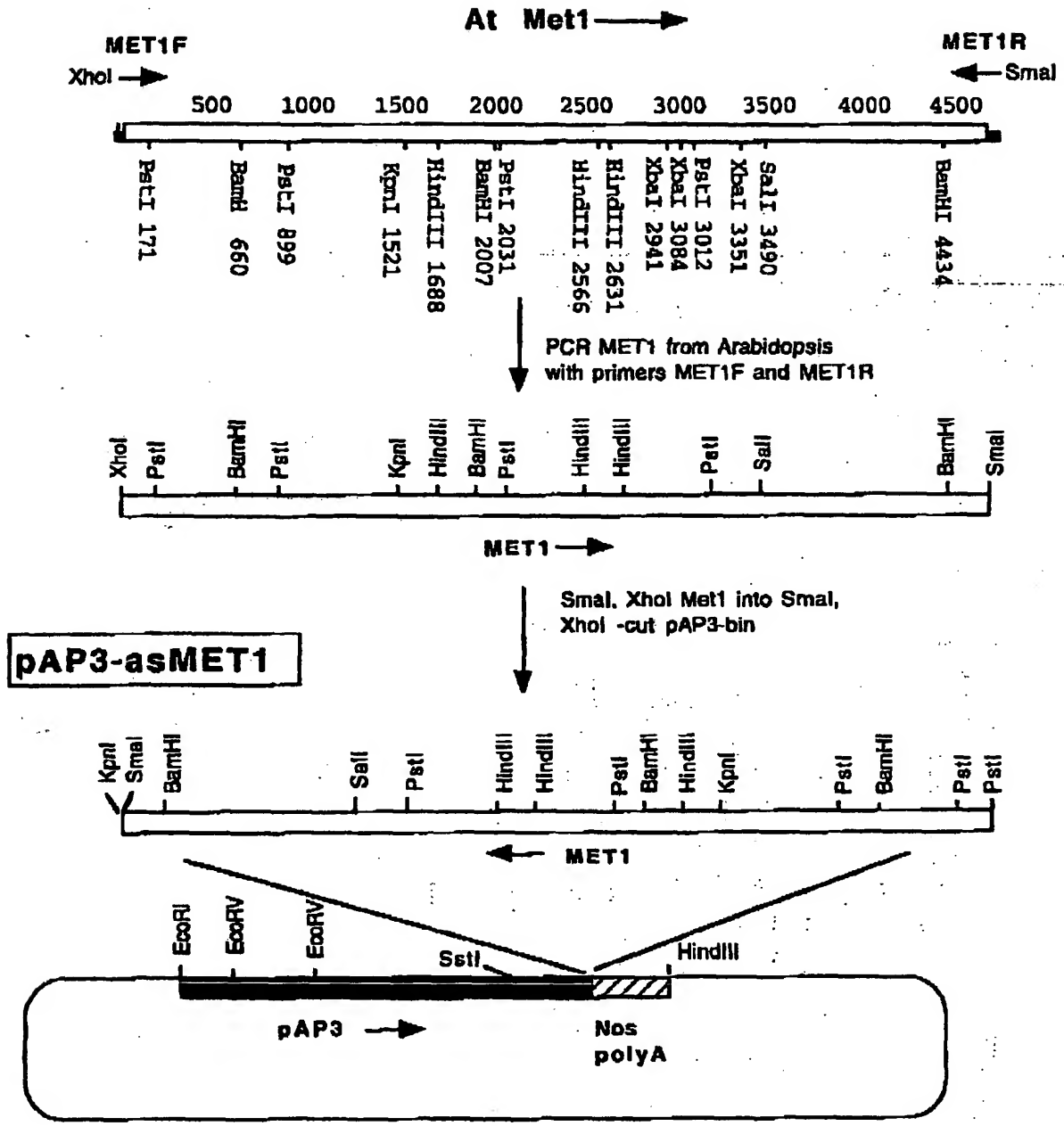

At Met1
MET1F
XhoI
MET1R
SmaI
500 1000 1500 2000 2500 3000 3500 4000 4500
PstI 171
BamH 660
PstI 869
KpnI 1521
HindIII 1688
BamHI 2007
PstI 2011
HindIII 2566
HindIII 2631
XbaI 2941
XbaI 3084
PstI 3012
XbaI 3351
SalI 3490
BamHI 4434
PCR MET1 from Arabidopsis
with primers MET1F and MET1R
XhoI PstI BamHI PstI KpnI HindIII BamHI PstI HindIII HindIII PstI SalI BamHI SmaI
MET1
SmaI, XhoI Met1 into SmaI,
XhoI -cut pAGL5-bin
pAGL5-asMET1
KpnI SmaI BamHI SalI PstI HindIII HindIII PstI BamHI HindIII KpnI PstI BamHI PstI PstI
MET1
EcoRI
HindIII
pAGL5
Nos
polyA

FIGURE 7


At Met1
MET1F
XhoI
MET1R
SmaI
500
1000
1500
2000
2500
3000
3500
4000
4500
PstI 171
BamHI 690
PstI 899
KpnI 1521
HindIII 1688
PstI 2031
BamHI 2007
HindIII 2566
HindIII 2631
XbaI 3084
XbaI 2941
PstI 3012
XbaI 3351
SalI 3460
BamHI 4434
PCR MET1 from Arabidopsis
with primers MET1F and MET1R
XhoI
PstI
BamHI
PstI
KpnI
HindIII
BamHI
PstI
HindIII
HindIII
PstI
SalI
BamHI
SmaI
MET1
SmaI, XhoI Met1 into SmaI,
XhoI -cut pAP3-bin
pAP3-asMET1
KpnI
SmaI
BamHI
SalI
PstI
HindIII
HindIII
PstI
BamHI
HindIII
KpnI
PstI
BamHI
PstI
PstI
MET1
EcoRI
EcoRV
EcoRV
SstI
HindIII
pAP3
Nos
polyA